Indian Journal of Fundamental and Applied Life Sciences ISSN: 2231– 6345 (Online)
An Open Access, Online International Journal Available at http://www.cibtech.org/jls.htm
2016 Vol. 6 (2) April-June, pp. 74-83/Taher and Moradshahi
Research Article

INVESTIGATION ON THE TOTAL REDUCING CAPACITY AND SILVER NANOPARTICLES SYNTHETIC POTENTIAL OF SOME PLANT SPECIES

Bahareh Taher and *Ali Moradshahi
Department of Biology, Faculty of Sciences, Shiraz University, 71454 Shiraz, Islamic Republic of Iran
**Author for Correspondence*

ABSTRACT
Plants with high total reducing capacity are expected to have high potency in silver nanoparticles (Ag NPs) synthesis. In the present study, free radical scavenging potential, total reducing capacity and Ag NPs synthetic potential of the methanolic leaf extracts of eight plant species were investigated. Two electron-transfer (ET) reaction- based assays, namely, 1,1-diphenyl-2-picryl-hydrazyl (DPPH) and Folin-Ciocalteu (F-C), were used for free radical scavenging and total reducing capacity determinations, respectively. Free radical scavenging potential expressed as µmol Trolox equivalent g^{-1} DW (µM TE g^{-1} DW), % inhibition of DPPH and IC_{50} (mg ml^{-1}) ranged from 14.23 to 0.11, 59.96 to 2.49 and 0.8 to 21.53, respectively. *Rosa persica*, *Elaeagnus angustifolia* and *Rubus armeniacus* showed the highest radical scavenging potential. Total reducing capacity ranged from 0.7 to 2.0 mg gallic acid equivalent g^{-1} DW (mg GAE g^{-1} DW). The order of plants for reducing capacity was similar, but not identical with the order of plants for free radical scavenging potential. Similar plant order was obtained for Ag NPs synthesis, which shows plants with high reducing capacity are more efficient in Ag NPs synthesis. When Ag NPs synthesis was examined as an ET-based assay for total reducing capacity determination, absorbance at 410 nm increased with the increase in extract concentration and, in most cases, the responses were linear in the concentration ranges tested. It is concluded that plants with high reducing capacity also have high free radical scavenging potential and their reducing capacity can be exploited for commercial green synthesis of metallic nanoparticles.

***Keywords*:** *Free Radical Scavenging Potential, Total Reducing Capacity, Ag NPs Synthesis, Correlation*

INTRODUCTION
Due to a wide medical and industrial applications of silver nanoparticles (Ag NPs) and also due to the problems associated with their physical and chemical production, green and eco-friendly synthesis of Ag NPs have become the focus of much research studies in recent years (Park *et al.*, 2011; Song *et al*., 2009; Thakkar *et al*., 2009). Ag NPs have applications in various fields such as biomedical (Becker, 1999), electronics (Wang *et al.*, 2010), spectroscopy (Haes *et al*., 2005) and chemical sensing (Murphy *et al*., 2008). As have been reported by many investigators, chemical protocols for the synthesis of Ag NPs usually involve toxic substances with adverse effects on the environment and human health (Song *et al*., 2009; Christensen *et al*., 2011). The drawback of producing metallic nanoparticles by physical methods is the high energy cost and low production rate (Li *et al*., 1999). Searching for nontoxic, inexpensive and eco-friendly procedures for metallic nanoparticles synthesis, the biological approach has received considerable attention as a suitable alternative to chemical and physical methods (Iravani *et al*., 2011; Leung *et al*., 2010; Saravanan *et al*., 2010; Shahverdi *et al*., 2007; Shankar *et al*., 2003).
Among many organisms and natural products, plant extracts with high antioxidant activity (Mansour *et al*., 2013; Ranjbar Nedamani *et al*., 2014; Guo *et al*., 2014) and the phytochemicals present in the extracts represent excellent scaffolds for the reduction and stabilization of metallic nanoparticles (Park *et al*., 2011; Vigneshwaran *et al*., 2006; Nune *et al*., 2009). Neem (*Azadirachta indica*) and mango (*Mangifera indica*) leaf extracts have been effectively used in the synthesis of Ag NPs (Shankar *et al*., 2004; Philip, 2011). Green tea (*Camellia sinensis*) and black tea leaf extracts have been used for Ag NPs synthesis at ambient conditions (Vilchis *et al*., 2008; Begum *et al*., 2009). It has been suggested that phenolic compounds in the extracts have been responsible for the reduction and stabilization of the synthesized

nanoparticles. In fact, many plant products such as polysaccharides, phenolic compounds and vitamins have been employed as reducing agents in metallic nanoparticles synthesis (Martinez-Castanon *et al*., 2008; Szydlowska-Czerniak *et al*., 2012; Kora *et al*., 2012). Sucrose and fructose can function as reducing agents for the synthesis of aqueous dispersions of Ag NPs (Mehta *et al*., 2010). Ag NPs with particle mean size of 5.3 nm were synthesized by using glucose as reducing agent (Park *et al*., 2011). Stable Ag NPs in the size ranges of 10.0 to 34 nm have been obtained using soluble starch as both reducing and stabilizing agent (Vigneshwaran *et al*., 2006). Gallic acid, a phenolic compound, has been used as reducing and coating agent in Ag NPs synthesis (Martinez-Castanon *et al*., 2008).

Different assay methods have been developed for the estimation of total reducing capacity and free radical scavenging potential of a sample (Marxen *et al*., 2007; Everette *et al*., 2010)**.** In general, theses assays are broadly classified into electron transfer (ET)-based and hydrogen atom transfer (HAT)-based. ET-based assays, such as FRAP and Folin-Ciocalteu (F-C) assays, involve one oxidation reduction (redox) reaction in which an electron is transferred from the antioxidant to the oxidant (Huang *et al*., 2005).

Synthesis of metallic nanoparticles also requires transfer of electron from a reductant to metal ion. In this process metal ion is reduced to zero-valent metallic nanoparticles (Szydlowska-Czerniak *et al*., 2012).

The concept of green synthesis of metallic nanoparticles using biological sources, such as higher plants and algae, is mainly based on the reducing capacity of these organisms (Vivekanandhan *et al*., 2009; Shankar *et al*., 2004; Philip, 2011). Indeed, together with methods such as FRAP, DPPH and F-C assays, metallic nanoparticle synthesis has been suggested as another ET-based assay for estimating antioxidant potential (more specifically, total reducing capacity) of chemical compounds and organisms (Szydlowska-Czerniak *et al*., 2012).

In the present study, free radical scavenging potential and total reducing capacity of methanolic leaf extracts of 12 plant species were determined by the DPPH and F-C assays and the correlation between these two methods was calculated. Then, the methanolic leaf extracts were used for green synthesis of Ag NPs and the correlations between free radical scavenging potential of the extracts, their total reducing capacities and Ag NPs synthetic potential were analyzed. Finally, the extracts potentials for Ag NPs synthesis as another ET-based assay were evaluated.

MATERIALS AND METHODS

Reagents and Plant Materials

All reagents were of analytical grade and were purchased from Sigma–Aldrich Chemical Co. (Darmstadt, Germany). Aerial parts of plants were collected in August 2013 from Yasoj (latitude 30.31 N, longitude 51.31E), Iran. Plants materials were washed and then dried at room temperature in the dark.

Extraction Procedure

One gram of powdered leaves of each plant was extracted with 30 ml methanol in 100 ml Erlenmeyer flask placed on a shaker (3000 rpm) for one day at room temperature in the dark. The extracts were centrifuged at 4000 rpm for 10 min and the supernatants were used for their antioxidants and total reducing capacity determination (Zamani and Moradshahi, 2014).

Free Radical Scavenging Capacity Analysis by DPPH Assay

Methanolic solution of DPPH radical is stable and has an absorbance maximum at about 515-520 nm. When reduced, the decrease in absorbance at 515 nm is proportional to the free radical scavenging capacity of the plant extract. The DPPH assay was performed according to the procedure described by Thaipong *et al*., (2006). In brief, 150 µL of standard solution or samples were added to 2850 µL DPPH solution and kept in the dark for 60 min after which the solutions absorbance was measured at 515 nm. Calibration curve was constructed using trolox in the concentration range of 25 to 800 µm in methanol. Free radical scavenging capacity is reported as µmol trolox equivalent per gram plants dry weight (µmol TE g^{-1} DW). The percent inhibition of the DPPH radical by one ml of the extracts was calculated according to the following equation: % inhibition = $[(A_c - A_s) / A_c] \times 100$

Where A_c is the absorbance of control at 515 nm and A_s is the absorbance of sample after 60 min incubation. The concentration of methanolic extract that scavenged 50 % of the DPPH radicals (IC_{50}) was calculated from the plot of the DPPH absorbance at 515 nm versus extract concentration.

Total Reducing Capacity Determination

Total reducing capacity of the extracts was determined spectrophotometerically using the Folin-Ciocalteu (F-C) assay. In this assay, it is believed that molybdenum VI in the complex is reduced to Mo (V) by electron transfer from the antioxidants (reductants). The assay was carried out as described by Velioglu *et al.*, (1998) with slight modification. In brief, 200 µL of standard solutions or samples were mixed with 1.5 mL of Folin-Ciocalteu reagent which was previously diluted ten folds with distilled water. After 5 min, 1.5 mL of 6 % (w/v) sodium bicarbonate solution was added to the solution. The mixture was kept for 90 min at room temperature and the absorbance was reordered at 750 nm. Calibration curve was constructed using gallic acid as the standard in the concentration range of 25-200 µg mL^{-1}. Total reducing capacity is reported as mg gallic acid equivalent per gram dry weight (mg GAE g^{-1} DW).

Green Synthesis of Ag NPs

Leaf extract was prepared by boiling one gram of powdered leaf in 30 ml deionized water for 5 min. The extract was cooled, filtered and then centrifuged at 3000 g for 15 min. The supernatant was diluted with deionized water to give 1.0 to 30 mg mL^{-1} of the original plant extract and used for Ag NPs synthesis. Typically, 100 µl of the sample was added to 2 ml of 10 mM $AgNO_3$ at ambient temperature and mixed thoroughly. After one hr, synthesis of Ag NPs was followed by measuring the solutions absorbance at 410 nm using double beam spectrophotometer (SHIMADZU 160A).

Statistical Analysis

Three replicates of each treatment sample were used for statistical analysis. Data are presented as means ± SE and means were compared using SPSS version 16. Duncan's multiple range test was used to determine significant differences at $p < 0.05$. Correlation analysis was carried out using Pearson's correlation and regression analysis using SPSS version 16.0.

RESULTS AND DISCUSSION

Free Radical Scavenging Capacity of the Extracts

Free radical scavenging capacity, percent inhibition of DPPH radicals and IC_{50} of the leaves extracts from 12 different plant species are presented in Table 1. Based on the free radical scavenging capacity, the extracts could be divided into three groups: 1) *Rosa persica*, *Elaeagnus angustifolia* and *Rubus armeniacus* having the highest scavenging potentials of 14.23, 12.72 and 9.52 µmol TE g^{-1} DW, respectively; 2) *Cyperus esculentus*, *Glycyrrhiza glabra*, *Plantago major*, *Echinochloa crus-galli* and *Mentha spicata* with antioxidant potentials ranging from 4.03 to 1.55 µmol TE g^{-1} DW and 3) *Solanum dulcamara*, *Sorghum bicolor*, *Physalis alkekengi* and *Amaranthus retroflexus* with less than 0.7 µmol TE g^{-1} DW were put together as the third group.

The plots of absorbance of DPPH radical at 515 nm versus extract concentration were linear for all the plants extracts in the range tested with $R^2 > 0.98$ which are shown for the plants *Rosa persica* and *Mentha spicata* in Figure 1. For *Rosa persica* about 1.5 mg mL^{-1} of extract was required to decrease the absorbance at 515 nm from about 1.04 to 0.17 whereas in the case of *Mentha spicata* about 8.5 mg mL^{-1} of extract was needed for the same reduction in absorbance at 515 nm. The percent inhibition of DPPH radical followed the same pattern as the radical scavenging capacity (Table 1). IC_{50}, which is inversely related to both free radical scavenging capacity and percent inhibition of DPPH radical, was 0.8 mg mL^{-1} for *Rosa persica* while for *Amaranthus retroflexus* 21.53 mg mL^{-1} of the original extract was needed to give 50 % inhibition of DPPH radicals.

Total Reducing Capacity of the Extracts

The first two groups with relatively high free radical scavenging potential were used for total reducing capacity determination by F-C assay which also gives an estimate of total phenolics content. As shown in Table 2, total reducing capacity ranged from 8.7 to 2.0 mg GAE g^{-1} DW with *Rosa persica* and *Rubus armeniacus* having the highest reducing compounds. The order of total reducing capacity of the extracts

was not exactly identical to the order of free radical scavenging potential of the extracts, but closely followed that order. The plots of absorbance at 750 nm versus extract concentrations were linear for all the plants extracts in the range tested with $R^2 > 0.99$ which are shown for *Rosa persica* and *Mentha spicata* in Figure 2. For *Rosa persica* and *Mentha spicata* 5.0 and 30.0 mg mL $^{-1}$ of the extracts were required to increase the absorbance at 750 nm from about zero to 1.7, respectively, indicating the presence of reducing substances in *Rosa persica* being 4 times higher as compared to *Mentha spicata*.

Table 1: Free Radical Scavenging Capacity, Percent Inhibition of DPPH Radicals and IC_{50} of Methanolic Leaf Extracts of 12 Plant Species. Each Value is Mean ± SE. In each Column, Values with Different Letter are Significantly Different at *P < 0.05*

Plant Material	µM TE g^{-1} DW	% Inhibition of DPPH	IC_{50} (mg ml^{-1})
Rosa Persica	14.23±0.512^{a}	59.96±2.079%^{a}	0.8±0.047^{a}
Elaeagnus Angustifolia	12.72±0.101^{b}	53.11±0.421%^{b}	0.98±0.017^{a}
Rubus Armeniacus	9.52±0.011^{c}	40.69±0.054%^{c}	1.24±0.012^{b}
Cyperus Esculentus	4.03±0.305^{d}	19.41±1.221%^{d}	3.30±0.109^{c}
Glycyrrhiza Glabra	3.81±0.198^{d}	17.86±0.798%^{d}	3.41±0.017^{c}
Plantago Major	2.82±0.133^{e}	10.86±0.560%^{e}	4.71±0.046^{d}
Echinochloa Crus-Galli	1.86±0.050^{f}	9.50±0.208%^{e}	6.12±0.263^{e}
Mentha spicata	1.55±0.040^{f}	9.12±0.164%^{e}	5.51±0.015^{f}
Solanum Dulcamara	0.70±0.055^{g}	4.43±0.227%^{f}	8.69±0.031^{g}
Sorghum Bicolor	0.66±0.066^{g}	4.69±0.260%^{f}	8.37±0.020^{g}
Physalis Alkekengi	0.56±0.034^{g}	4.13±0.139%^{f}	15.54±0.07^{h}
Amaranthus Retroflexus	0.11±0.065^{g}	2.49±0.369%^{f}	21.53±0.023^{i}

Table 2: Total Reducing Capacity and Ag NPs Synthetic Potential of Methanolic Leaf Extracts of Eight Plant Species. Each Value is Mean± SE. Values with Different Letter are Significantly Different at *P < 0.05*. Ag NPs Production by 10 mg ml^{-1} of the Extract was Compared after One hr Incubation

Plant Material	Total Reducing Capacity (mg GAE g^{-1} DW)	AgNPs Synthesis (Absorbance at 410 nm)
Rosa Persica	8.7±0.33^{a}	1.66±0.07^{b}
Rubus Armeniacus	6.8±0.29^{b}	2.00±0.00^{a}
Elaeagnus Angustifolia	5.0±0.11^{c}	2.00±0.00^{a}
Glycyrrhiza Glabra	4.5±0.16^{c}	0.83±0.02^{c}
Plantago Major	3.3±0.17^{d}	0.75±0.02^{c}
Cyperus Esculentus	2.5±0.00^{e}	0.63±0.01^{d}
Mentha Spicata	2.5±0.05^{e}	0.68±0.03^{d}
Echinochloa Crus-Galli	2.0±0.08^{e}	0.29±0.01^{e}

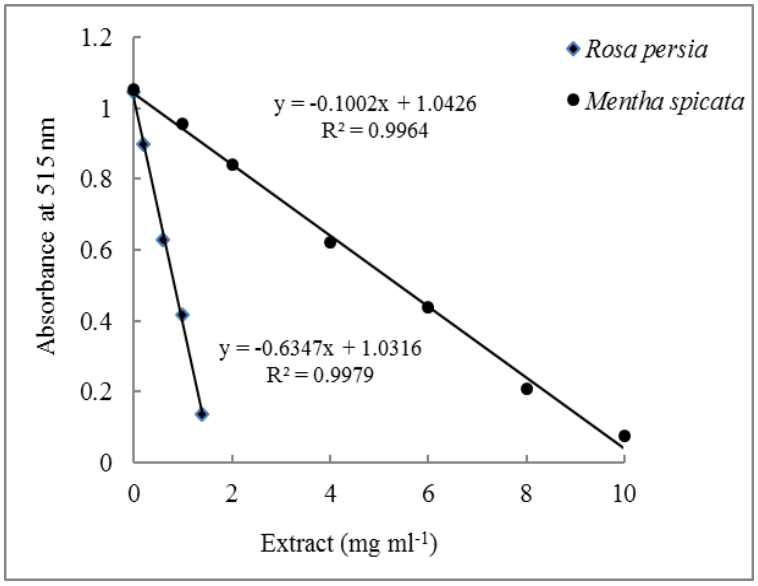

Figure 1: DPPH Absorbance at 515 nm as Affected by Different Concentrations of Methanolic Leaf Extracts of *Rosa Persica* and *Mentha Spicata*. Decrease in Absorbance at 515 nm is due to Reduction of DPPH Radical to DPPH-H

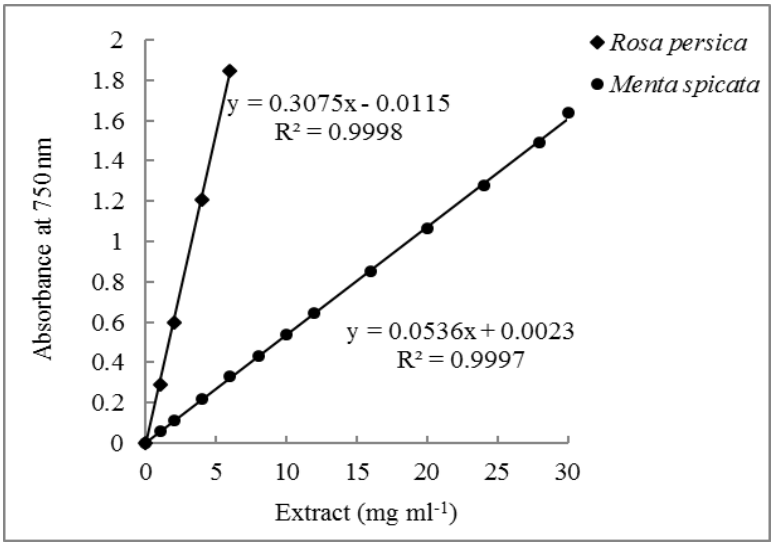

Figure 2: Absorbance of Folin-Ciocalteu Reagent at 750 nm as Affected by Different Concentrations of Methalonic Leaf Extracts of *Rosa Persica* and *Mentha Spicata*. Increase in Absorbance at 750 nm is due to Probable Reduction to Mo (VI) to Mo (V)

Green Synthesis of Silver Nanoparticles

Since surface plasmon resonance peak of Ag NPs occurs at about 410 nm, green synthesis of Ag NPs by the leaves extracts was monitored at this wavelength using UV–VIS spectrophotometer. The leaves extracts order for Ag NPs synthesis was not identical with the order of leaves extracts for free radical scavenging potential or for total reducing capacity. But, the extracts of *Rosa persica*, *Elaeagnus angustifolia* and *Rubus armeniacus* with the highest free radical scavenging and reducing capacity, were more efficient in synthesizing Ag NPs as compared to the other leaves extracts (Table 2). As shown for *Rosa persica* and *Mentha spicata* in Figure 3, absorbance at 410 nm, which is indicative of Ag NPs production, increased with the increase in extract concentrations. The increase in absorbance at 410 nm

correlated well with extract concentrations, and in most cases, linear relationship was obtained between the absorbance at 410 nm and extract concentrations. In some cases, the relationship between absorbance and extract concentration tended to be hyperbolic with high intercept values which needs to be resolved before using Ag NPs synthesis as an ET-based assay for total reducing capacity determination.

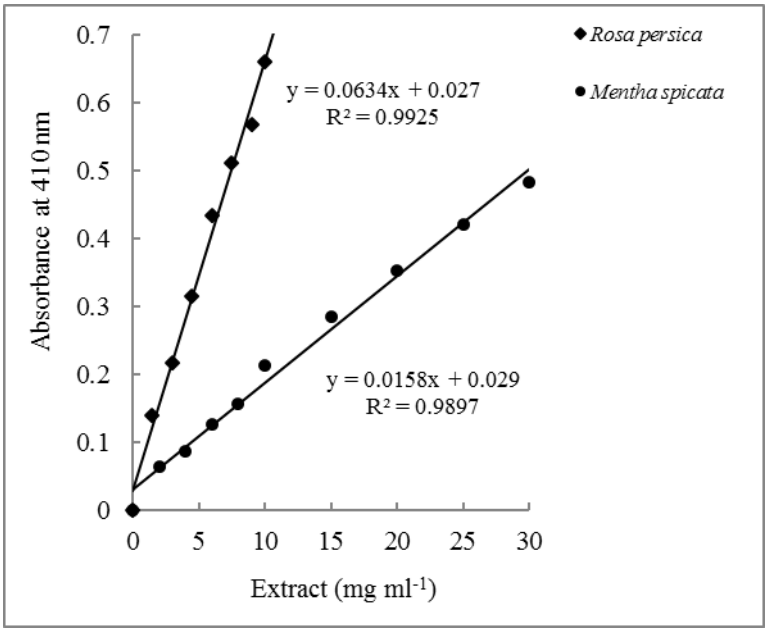

Figure 3: Relationship between Absorbance at 410 nm and Leaf Extract Concentration of *Rosa Persica* and *Mentha Spicata* after One hr Incubation

Correlations

The correlation coefficients (R^2) between the free radical scavenging potential, total reducing capacity and Ag NPs synthesis potential of eight plant leaves extracts are shown in Figure 4. The correlation between the free radical scavenging potential and total reducing capacity was $R^2 = 0.7672$ indicating that 76 % of the free radical scavenging potential of the leaves extracts is contributed by reducing compounds such as plant phenolics. The correlation between free radical scavenging capacity and Ag NPs synthesis was high ($R^2 = 0.8218$) suggesting that components contributing to free radical scavenging potential are mainly responsible for the Ag NPs synthesis. The correlation coefficient of R^2=0.674 between the total reducing capacity and Ag NPs synthesis potential indicates that reducing compounds in the extracts, such as plant phenolics, are involved in the reducing of Ag^{+1} to Ag NPs. In addition, other reducing components of the extracts, not detectable by F-C assay, may also contribute to Ag NPs synthesis.

The use of living organisms and biologically derived natural products are the eco-friendly alternatives to the physical and chemical methods for metallic nanoparticles synthesis (Kim and Song, 2009). Several plant extracts have been investigated for their potential in the reduction of silver ion to Ag NPs (Thakkar *et al*., 2009). With *Murraya koenigii* leaves extract, Ag NPs formation is reported to be rapid and the rate of reduction increased with the increase in extract concentrations (Christensen *et al*., 2011). The extracts from alfalfa, lemon grass and green tea which served as both the reducing and capping agents have been used in the synthesis of silver and gold nanoparticles (Gardea Torresdey *et al*., 2003; Shankar *et al*., 2005; Smuleac *et al.*, 2011). Plant-based exudate gums such as gum acacia (Dhar *et al*., 2008) and gum kondago (Kora *et al.*, 2010) have been utilized as both reducing and stabilizing agents for Ag NPs synthesis. Monodispersed and size controlled spherical Ag NPs of around 5.7 nm has been obtained using gum ghatti (Kora *et al*., 2012). Results of the present study clearly indicate that plants with higher reducing capacity are more potent in Ag NPs synthesis. Therefore, search for plants with high reduction potential makes green synthesis of metallic NPs more cost efficient.

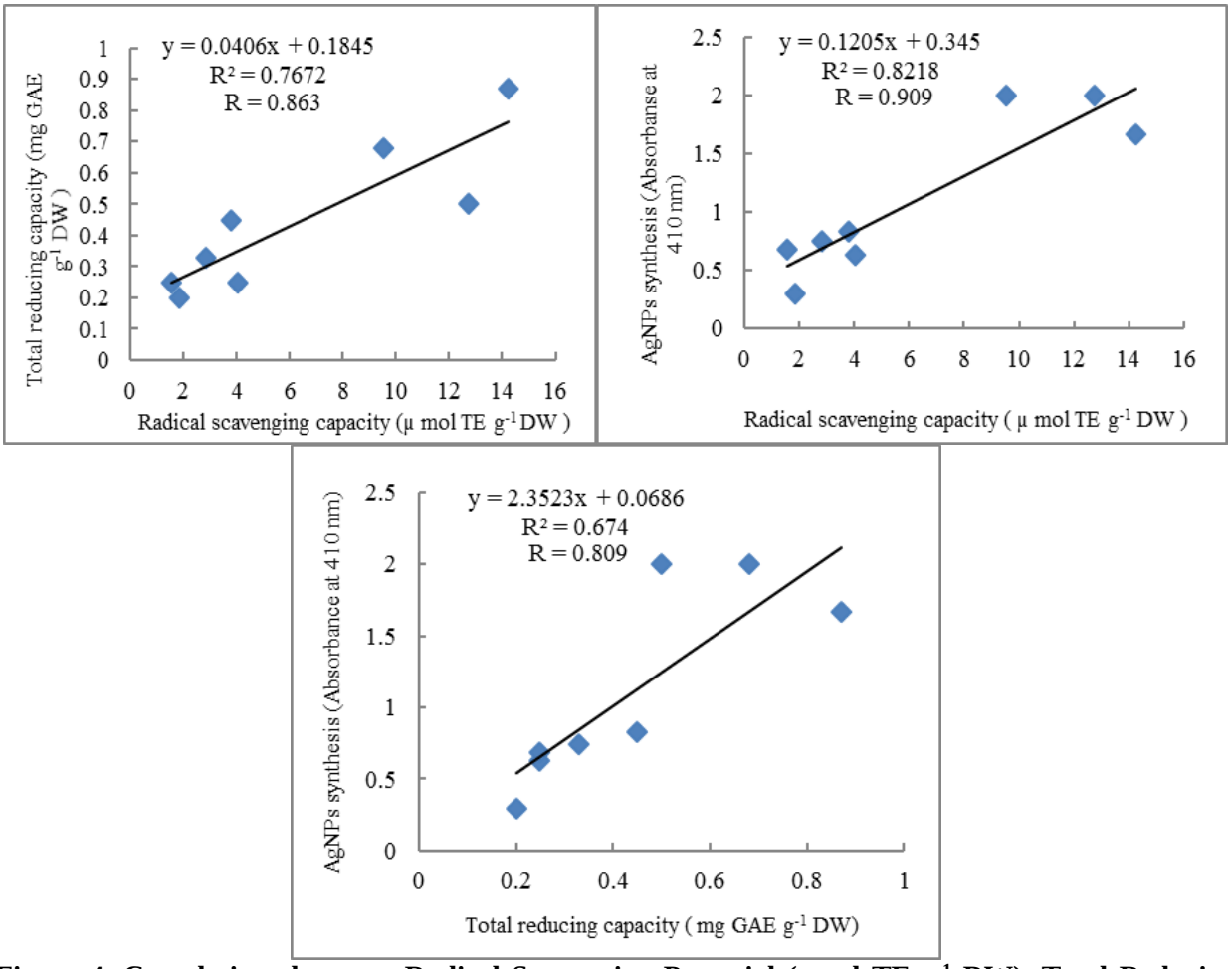

Figure 4: **Correlations between Radical Scavenging Potential (µmol TE g^{-1} DW), Total Reducing Capacity (mg GAE g^{-1} DW) and Ag NPs Synthetic Potential (Absorbance at 410 nm) of Eight Plant Species Leaf Extracts**

A silver nanoparticle-based assay for antioxidant potential (total reducing capacity) determination has been proposed by Szydlowska-Czerniak *et al.*, (2012). Significant positive correlations (r = 0.7564-0.8516, *p<0.001*) between the Ag NPs assay with FRAP, DPPH and F-C assays were observed for the rape seed extracts.

The absorbances versus concentrations of sinapic acid, gallic acid, caffeic acid, quercetin and ascorbic acid, as antioxidant standards, were linear in the concentration ranges tested with quercetin having the lowest intercept value.

They did not examine the linearity of assay for rapeseed or other plant extracts. In the present study, with most extracts, linear relationships were obtained between absorbance at 410 nm and extracts concentrations as shown for the plants *Rosa persica* and *Mentha spicata* in Figure 3. In some cases the relationship tended to be hyperbolic (data not shown). As stated by Szydlowska-Czerniak *et al.*, (2012), the Ag NPs-based assay method for antioxidant potential (total reducing capacity) determination is inexpensive, eco-friendly and simpler compared to other ET-based assays. More experiments are needed to conclusively establish the linearity of assay when Ag NPs synthesis is used for total reducing capacity determination of various plants extracts.

ACKNOWLEDGEMENT
The authors would like to thank Shiraz University Research Council for financial support of this research.

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